

## Video Article

# Bacterial Gene Expression Analysis Using Microarrays

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## Abstract

## Protocol

### Reverse Transcriptase Reaction

Turn heating block to 70-80°C and 42°C. Water baths can also be used in this step. If using heating blocks, put some water so that the heat is uniform.

1. Priming reaction
  1. Take 3 µg of RNA
  2. Adjust volume to 13 µl with RNase free water
  3. Add 2.5 µl of random hexamer primers (2mg/mL)
2. Mix well and incubate at 70-80°C for 8 minutes. Then, place on ice for 5 min.
3. Prepare RT cocktail (14.5 µl/ rxn):

Component	Volume
5X First Strand buffer	6 µl
0.1 M DTT	3 µl
25X aminoallyl-dNTP mix	1.2 µl
SuperScript II RT (200U/µl)	2.3 µl
Water	2.0 µl

\* For n reactions, prepare Master Mix for n+0.5 aliquots of cocktail to assure that you will not run out.

4. Add 14.5 µl to cooled reactions.
5. Mix (do not vortex) and incubate at 42°C for 3-4 hours
6. Samples can be stored at -20°C if necessary.

### Hydrolyze RNA

1. To each sample, add 3 µl of 1 N NaOH and 0.6 µl of 0.5 M EDTA. (Final conc.s = 100mM NaOH, 10mM EDTA)
2. Mix and incubate at 65°C for 10 minutes.
3. Neutralize by adding 33.6 µl 1M HEPES, pH=7.0 (final conc.= 500mM HEPES)
4. Samples can be stored at -20°C, if necessary.

### Reaction Purification: Removal of unincorporated aa-dUTP and free amines

**Note:** This purification protocol is modified from the Qiagen QIAquick PCRpurification kit protocol. The phosphate wash and elution buffers are substituted for the Qiagen supplied buffers because the Qiagen buffers contain free amines that compete with the Cy dye coupling reaction. The columns from the mini-prep kit can also be used.

1. Mix cDNA reaction with 300 µl (or 5X reaction volume = 336 µl) buffer PB (Qiagen supplied) and transfer to QIAquick column.
2. Place the column in a 2 ml collection tube (Qiagen supplied) and centrifuge at ~13,000 rpm for 1 minute. Empty collection tube.
3. To wash, add 750 µl phosphate wash buffer (not Qiagen's) to the column and centrifuge at ~13,000 rpm for 1 minute.
4. Empty the collection tube and repeat the 750 µl wash and centrifugation step.
5. Empty the collection tube and centrifuge the column an additional 1 minute at maximum speed.
6. Transfer column to a new 1.5 mL microfuge tube and carefully add 30 µl phosphate elution buffer. (see solution preparation section)
7. Incubate for 1 minute at room temperature.
8. Elute by centrifugation at ~13,000 rpm for 1 minute.
9. Elute a second time (with another 30 µl of phosphate elution buffer) into the same tube by repeating steps 6-8. The final elution volume should be ~60 µl.
10. The amount of cDNA can be quantified at this stage:  

$$\text{ng cDNA} = (\text{dilution factor})(\text{OD}_{260})(37)(\text{total volume eluted from column})$$
11. Dry sample in a speed vac at 45°C.
12. Samples can be stored at -20°C if necessary.

## Coupling aa-cDNA to Cy Dye Ester

1. Resuspend aminoallyl-labeled cDNA with 10  $\mu$ L 50 mM Na-bicarbonate, pH=9.0 if necessary. (This buffer should be made fresh every two weeks)
2. Working in a dark place, add the probe to a tube of the appropriate Cy dye. Pipet up and down several times to resuspend the dye.
3. Incubate the reaction for 1 hour in the dark at room temperature. (Incubation can go up to two hours)

## Reaction Purification II: Removal of uncoupled dye

1. To the reaction add 35  $\mu$ L 100 mM NaOAc pH 5.2. Mix thoroughly.
2. Add 250  $\mu$ L ( or 5X reaction volume = 225  $\mu$ L) Buffer PB (Qiagen supplied) to each sample and mix by pipetting up and down.
3. Place a QIAquick spin column in a 2 mL collection tube (Qiagen supplied), apply the sample to the column, and centrifuge at ~13,000 for 1 minute. Empty collection tube.
4. To wash, add 0.75 mL Buffer PE (Qiagen supplied) to the column and centrifuge at ~13,000 for 1 minute.
5. Repeat step 4.
6. Empty collection tube and centrifuge column for an additional 1 minute at maximum speed.
7. Place column in a clean 1.5 mL microfuge tube and carefully add 30  $\mu$ L Buffer EB (Qiagen supplied) to the center of the column membrane.
8. Incubate for 1 minute at room temperature.
9. Elute by centrifugation at ~13,000 rpm for 1 minute.
10. Elute a second time into the same tube by repeating steps 7-9. The final eluted volume should be ~60  $\mu$ L.

## Analysis of Labeling Reaction

The amount of cDNA and label can be quantified at this stage:

- Measure OD 260, 550 and 650 (Blank is water.) Use nanodrop microarray program.
- Calculate the pmol of dyes incorporation and pmol DNA and nuc/dye ratio with given equations below:

$$\text{pmol nucleotides} = [\text{OD260} * \text{volume} (\mu\text{L}) * 37 \text{ ng}/\mu\text{L} * 1000 \text{ pg/ng}] / 324.5 \text{ pg/pmol}$$

*Note:* 1 OD260 = 37 ng/ $\mu$ L for cDNA; 324.5 pg/pmol average molecular weight of a dNTP)

$$\text{pmol Cy3} = \text{OD550} * \text{volume} (\mu\text{L}) / 0.15$$

$$\text{pmol Cy5} = \text{OD650} * \text{volume} (\mu\text{L}) / 0.25$$

$$\text{nucleotides/dye ratio} = \text{pmol cDNA} / \text{pmol Cy dye}$$

If you are using nanodrop, it already gives pmol/ $\mu$ L for each dye. You only need to multiply this number by volume to get total pmol dye incorporation.

*Note:* >150-200 pmol of dye incorporation per sample and a ratio of less than 20 nucleotides/dye molecules is optimal for hybridizations.

- Samples can be stored at -20°C at dark.
  - Combine probes to be hybridized together and dry down in speedvac at 42°C.
  - Samples can be stored at -20°C until ready to hyb.

## Pre-hyb slides

1. Place slide face down over RT 100 mL water in a clean red slide holder for 3-5 minutes
2. Snap dry the slide by placing face up on a 100°C heating block for a few seconds - watch for the condensation to vanish
3. UV cross-link the slide at 250 mJoules ( Enter 2500 to the UV crosslinker)
4. Hydrate slides by dipping in pre-warmed 42°C water. Spin 5 min at 700 RPM at RT.
5. Incubate slides in 50ml Coplin jar containing pre-warmed pre-hyb (5XSSC, 1% BSA, 0.1% SDS) for at least 45 minutes @ 42°C, and incubation can go longer if necessary.  
50 mL pre-hyb buffer: 12.5 mL 20x SSC, 10 mL 5% BSA, 0.5 mL 10 % SDS, 27 mL water
6. Dip slides in pre-warmed water (42°C) and agitate a couple of minutes to remove pre-hyb buffer.
7. Rinse in isopropanol and spin 5 min at 700 rpm, room temp, to dry.

## Preparing samples for hybridization

1. Resuspend probe in water (volumes are given below)

For total vol. = 30 $\mu$ L	For total vol. = 35 $\mu$ L	For total vol.= 40 $\mu$ L	
19.8 $\mu$ L water	23.55 $\mu$ L water	27.2 $\mu$ L water	
1.5 $\mu$ L	1.5 $\mu$ L	1.5 $\mu$ L	10 mg/ml salmon sperm DNA (Invitrogen)
1.2 $\mu$ L	1.2 $\mu$ L	1.2 $\mu$ L	12.5 mg/ml tRNA
4.5 $\mu$ L	5.25 $\mu$ L	6 $\mu$ L	20XSSC (3X final)
3 $\mu$ L	3.5 $\mu$ L	4 $\mu$ L	1% SDS

*Note:* The order of addition of the reagents are important. Do not prepare master mix.

2. Boil for 5 minutes, then spin 5 minute at 14,000 rpm. Cool for 2 min at room temp.

## Hybridization

1. Add 10-12  $\mu$ l of 3X SSC to the wells of the hybridization chamber on each edge and in the middle.
2. Add a clean lifter slip to the appropriate area of the slide and gently load in hybridization solution (30 -40  $\mu$ l). There should be extra sample on either edge of the cover slip.
3. Place the slide in the chamber. Tighten the screws and place in a 65°C water bath overnight. Do not place the chamber directly on the bottom of the water bath. Place a block on top of the uppermost chamber to hold everything in place. Keep the lid on the water bath to protect the light sensitive samples.

## Hyb Washes

1. Prepare the following buffers:
  - 1X SSC, 0.05 % SDS
  - 0.06 X SSC
2. After the incubation unseal hybridization chamber. Remove the slide from the chamber, taking care not to disturb the coverslip.
3. To remove coverslips, submerge slides in a dish containing warm 1X SSC, 0.05 % SDS wash buffer coverslip facing the bottom of the dish. The coverslip will fall off by moving the slide side to side.
4. After the coverslip is removed, place the slide in a rack a fresh bath with warm 1XSSCm 0.05% SDS. Agitate for a couple of minutes
5. Transfer slides plus rack to a bath of 0.06 X SSC.
6. Transfer slides to another bath of 0.06 X SSC containing a fresh rack, blotting the slide on a paper towel to remove as much SDS-containing buffer as possible before placing in the fresh bath.
7. Agitate slides for a couple of minutes.
8. Spin immediately at room temperature for 5 min @ 700 rpm, then slides are ready to scan. Store in the dark until scanned.

## References

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